426-27 049 Class Subclass ISSUE CLASSIFICATION

[redacted]

5725895

5725895

UTILITY SERIAL NUMBER

PAT... MAY 10 1998

PATENT NUMBER

SERIAL NUMBER	FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
08/528,858	09/15/95	426	049	1302	Wong

APPLICANTS

JED W. FAHEY, ELDERSBURG, MD; PAUL TALALAY, BALTIMORE, MD.

CONTINUING DATA********
VERIFIED

MW

FOREIGN/PCT APPLICATIONS********
VERIFIED

MW

FOREIGN FILING LICENSE GRANTED 12/19/95 ***** SMALL ENTITY *****

Foreign priority claimed 35 USC 119 conditions met	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
☐ yes ☐ no ☐ yes ☐ no		MD	2	47	13	$2,084.00	46528/102 JD

Verified and Acknowledged Examiner's initials

ADDRESS

FOLEY AND LARDNER
3000 K STREET NW
SUITE 500
WASHINGTON DC 20007-5109

TITLE

METHOD OF PREPARING A FOOD PRODUCT FROM CRUCIFEROUS SEEDS

U.S. DEPT. OF COMM./ PAT. & TM—PTO-436L (Rev.12-94)

PARTS OF APPLICATION FILED SEPARATELY

Applications Examiner

NOTICE OF ALLOWANCE MAILED		CLAIMS ALLOWED	
8-14-97	Assistant Examiner	Total Claims: 16	Print Claim: 1

ISSUE FEE		DRAWING		
Amount Due: 645.00	Date Paid: 11/04	Sheets Drwg.: 2	Figs. Drwg.: 23	Print Fig.: None

LESLIE WONG
PRIMARY EXAMINER
GROUP 1300

Leslie Wong 8/13/97 Primary Examiner
PREPARED FOR ISSUE

ISSUE BATCH NUMBER: 043

Label Area

WARNING: The information disclosed herein may be restricted. Unauthorized disclosure may be prohibited by the United States Code Title 35, Sections 122, 181 and 368. Possession outside the U.S. Patent & Trademark Office is restricted to authorized employees and contractors only.

Form PTO-436A (Rev. 8/92)

ISSUE FEE IN FILE
1-B1

Exhibit 1-B(i)

08 528858

PATENT APPLICATION
08528858

APPROVED FOR LICENSE ☐

ALS OCT 3 09542

CONTENTS

Date Entered or Counted			Date Received or Mailed
	1.	Application 2 drw papers.	
	2.	Ent Small Entity	10-24-95
	3.	Disclosure statement	12-14-95
m 8-30-96	4.	Restriction (1)	9-3-96
	5.	Election	10-3-96
R 12-20-96	6.	Rej (3)	12-24-96
	7.	Examiner Interview Summary	3-11-97
	8.	Req RECON w/ Declaration	3-17-97
m 5-23-97	9.	Letter	5/23/97
	10.	Amdt A	6-18-97
	11.	Ltr. Assoc P/A	8-12-97
	12.	Examiner Interview Summary	8-14-97
m 8-14-97	13.	Exmr Amdt/B	8-14-97
~~11-21-97~~	14.	Letter	9-23-97
11-21-97	15.	Issue Fee	9-23-97
	16.	PTO Grant MAR 10 1998	
	17.	Ltr - IDS w/cert.	1-27-98
	18.	Ltr. Assoc P/A	1-27-98
	19.	Letter	3/2/98
	20.	IDS	10-18-99
	21.	IDS	4-30-99
	22.		
	23.		
	24.		
	25.		
	26.		
	27.		
	28.		
	29.		
	30.		
	31.		
	32.		

Staple Issue Slip Here

POSITION		ID NO.	DATE
CLASSIFIER		21	10/26/95
EXAMINER		249	11/14/95
TYPIST		335	12/19/95
VERIFIER		341	12/19/95
CORPS CORR.			
SPEC. HAND			
FILE MAINT.			
DRAFTING			

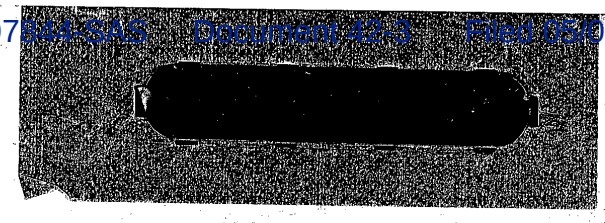
INDEX OF CLAIMS

Claim Final	Claim Original	Date 3/29/96	10/10	3/25/97	8/13
	1	*	N	N	
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	14				
	15				
	16				
	17				
	18				
	19				
	20		N	N	
1	21		✓	=	=
2	22				
3	23				
4	24				
5	25				
6	26		✓	-	=
	27		N	N	
7	28		✓	-	=
8	29				
9	30				
10	31				
11	32				
12	33				
13	34		✓	-	=
	35		N	N	
14	36		✓	=	=
15	37		✓	-	=
16	38		✓	-	=
	39		N	N	
	40				
	41				
	42				
	43				
	44				
	45				
	46				
	47	+	N	N	
	48				
	49				
	50				

SYMBOLS

- ✓ ..... Rejected
- = ... Allowed
- - (Through numberal) Canceled
- + ... Restricted
- N ..... Non-elected
- I ..... Interference
- A ..... Appeal
- O ..... Objected

Claim Final	Claim Original	Date
	51	
	52	
	53	
	54	
	55	
	56	
	57	
	58	
	59	
	60	
	61	
	62	
	63	
	64	
	65	
	66	
	67	
	68	
	69	
	70	
	71	
	72	
	73	
	74	
	75	
	76	
	77	
	78	
	79	
	80	
	81	
	82	
	83	
	84	
	85	
	86	
	87	
	88	
	89	
	90	
	91	
	92	
	93	
	94	
	95	
	96	
	97	
	98	
	99	
	100	



called 5/22
5/20

SEARCHED

Class	Sub.	Date	Exmr.
424	049	12/19/96	cwng
426	007 044 049 052 615 629 655 425 429 430 431	5/22/97	L.Wng
426	007 044 049 052 615 629 655 425 429 430 431	8/12/97	L.Wng

SEARCH NOTES

	Date	Exmr.
APS	12/19/96	cwng

INTERFERENCE SEARCHED

Class	Sub.	Date	Exmr.
426	049 052 615 425 429 431	8/13/97	L.Wng

1-B4
(RIGHT OUTSIDE)

BAR CODE LABEL

U.S. PATENT APPLICATION

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
08/528,858	09/15/95	426	1302

APPLICANT

JED W. FAHEY, ELDERSBURG, MD; PAUL TALALAY, BALTIMORE, MD.

CONTINUING DATA********
VERIFIED

_____

FOREIGN/PCT APPLICATIONS********
VERIFIED

_____

FOREIGN FILING LICENSE GRANTED 12/19/95

STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.
MD	2	47	13	$2,084.00	46528/102/JO

ADDRESS

FOLEY AND LARDNER
3000 K STREET NW
SUITE 500
WASHINGTON DC 20007-5109

TITLE

CANCER CHEMOPROTECTIVE FOOD PRODUCTS

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.

By authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

1-B5

Date Certifying Officer

08 528858

Law Offices
FOLEY & LARDNER
Suite 500
3000 K Street, N.W.
Washington, DC 20007-5109
(202) 672-5300

Assistant Commissioner for Patents
Washington, D. C. 20231

Sir:

Transmitted herewith for filing is the patent application of:

INVENTOR(S): Jed W. FAHEY, Paul TALALAY

TITLE: CANCER CHEMOPROTECTIVE FOOD PRODUCTS

In connection with this application, the following are enclosed:

44 Pages of Specification with Abstract

47 Claims

2 Sheets of Drawings

XX Declaration, Power of Attorney

XX Assignment to: **Johns Hopkins School of Medicine**

____ Certified Priority Application and Priority Claim

____ Statement of Small Entity Status

____ Other:

The fee has been calculated as shown below. (Small entity fees indicated in parentheses.)

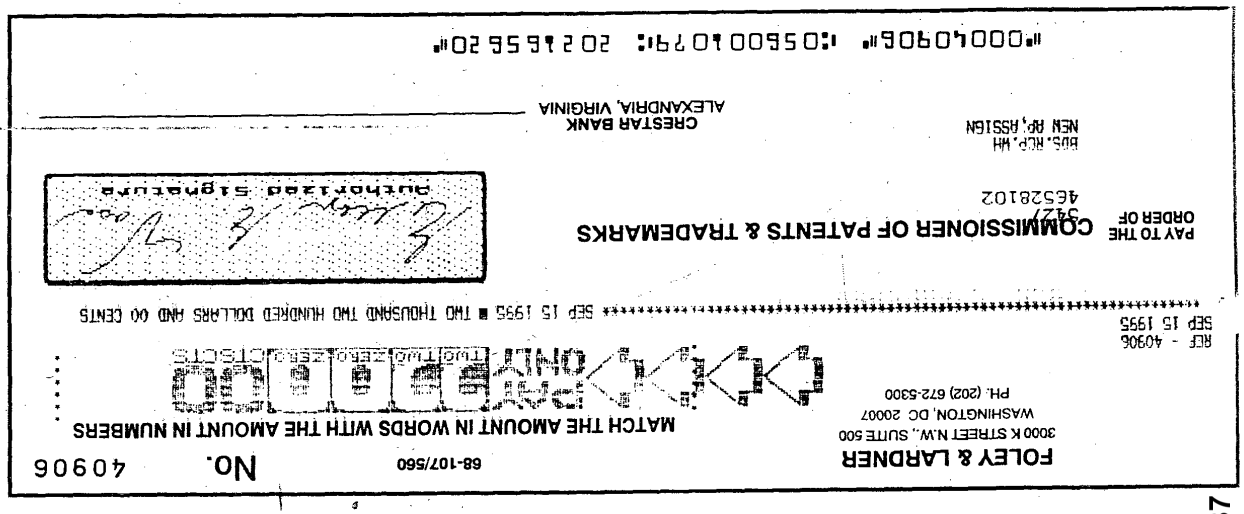
(1) For	(2) Number Filed	(3) Number Extra	(4) Rate	(5) Basic Fee $730 ($365)
Total Claims	47 - 20 =	27	x $22 (x $11)	594.00
Independent Claims	14 - 3 =	11	x $76 (x $38)	836.00
Multiple Dependent Claims			$240 ($120)	-
Assignment Recording Fee			$ 40	40.00
			TOTAL FEE:	$2,200.00

A check in the amount of the above TOTAL FEE is attached. This amount is believed to be correct; however, the Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 19-0741.

Respectfully submitted,

Date: September 15, 1995
Docket No.: 46528/102/JOHO

Bernhard D. Saxe
Reg. No. 28,665



1-B7

08 528858

FOLEY & LARDNER

September 15, 1995

46528/102/JOHO

The "Received" stamp of the U.S. Patent & Trademark Office hereon acknowledges receipt of the accompanying Patent Application of FAHEY *et al.*

for CANCER CHEMOPROTECTIVE FOOD PRODUCTS

Including:

44 pages of spec.; 47 claims; 2 shts. dwgs.; XX Declaration; XX Oath; XX Assignment; and Check for $2,200.00

Filed: September 15, 1995

Return to: BDS:tl:RCP:wh
Inspected by: ____

PATENT APPLICATION SERIAL NO. 08/528858

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

110 MG 10/11/95 08528858

1 101 2,160.00 CK 46528/102/JO

OK Refund $76.00 BP

SE18051 12/11/95 08528858

19-0741 180 101 76.00CR

PTO-1556
(5/87)

2

#2 08/528858 A

Inventors: Jed W. Fahey
Paul Talalay

METHOD OF PREPARING A FOOD PRODUCT FROM CRUCIFEROUS SEEDS

~~CANCER CHEMOPROTECTIVE FOOD PRODUCTS~~

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of grant PO1 CA 44530, entitled "Novel Strategies for Chemoprotection Against Cancer", (Paul Talalay, Principal Investigator) awarded by the National Cancer Institute, Department of Health and Human Services.

BACKGROUND OF THE INVENTION

I. Field of Invention

This invention relates to a dietary approach to reducing the level of carcinogens in animals and their cells and thereby reducing the risk of developing cancer. In particular, this invention relates to the production and consumption of foods which are rich in cancer chemoprotective compounds. More specifically, this invention relates to chemoprotective compounds that modulate mammalian enzymes which are involved in metabolism of carcinogens. This invention relates to food sources which are extremely rich in compounds that induce the activity of Phase 2 enzymes, without inducing biologically significant activities of those Phase 1 enzymes that activate carcinogens.

II. Background

It is widely recognized that diet plays a large role in controlling the risk of developing cancers and that increased consumption of fruits and vegetables reduces cancer incidence in humans. It is believed that a major mechanism of protection depends on the presence of chemical components in plants that, when delivered to

mammalian cells, elevate levels of Phase 2 enzymes that detoxify carcinogens.

Early studies on the mechanism of chemoprotection by certain chemicals assumed that these chemoprotectors induced activities of monooxygenases, also known as Phase 1 enzymes or cytochromes P-450. However, Talalay *et al.*, [reviewed in "Chemical Protection Against Cancer by Induction of Electrophile Detoxication (Phase II) Enzymes" In: CELLULAR AND MOLECULAR TARGETS OF CHEMOPREVENTION, L. Wattenberg *et al.*, CRC Press, Boca Raton, FL, pp 469-478 (1992)] determined that administration of the known chemoprotector butylated hydoxyanisole (BHA) to rodents resulted in little change in cytochromes P-450 (Phase 1 enzyme) activities, but profoundly elevated Phase 2 enzymes. Phase 2 enzymes such as glutathione transferases, NAD(P)H:quinone reductase (QR) and glucuronosyltransferases, detoxify DNA-damaging electrophilic forms of ultimate carcinogens. Selective inducers of Phase 2 enzymes are designated monofunctional inducers. Prochaska & Talalay, *Cancer Res.* 48: 4776-4782 (1988). The monofunctional inducers are nearly all electrophiles and belong to 8 distinct chemical classes including (1) diphenols, phenylenediamines and quinones; (2) Michael reaction acceptors containing olefins or acetylenes conjugated to electron-withdrawing groups; (3) isothiocyanates; (4) 1,2-dithiole-3-thiones; (5) hydroperoxides; (6) trivalent inorganic and organic arsenic derivatives; (7) heavy metals with potencies related to their affinities for thiol groups including Hg^{2+}, and Cd^{2+}; and (8) vicinal dimercaptans. Prestera *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2963-2969 (1993). The only apparent common property shared by all of these inducers is their ability to react with thiol groups.

Chemoprotective agents can be used to reduce the susceptibility of mammals to the toxic and neoplastic effects of carcinogens. These chemoprotectors can be of

plant origin or synthetic compounds. Synthetic analogs of naturally occurring inducers have also been generated and shown to block chemical carcinogenesis in animals. Posner *et al.*, *J. Med. Chem.* 37: 170-176 (1994); Zhang *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 3147-3150 (1994); Zhang *et al.*, *Cancer Res. (Suppl)* 54: 1976s-1981s (1994).

Highly efficient methods have been developed for measuring the potency of plant extracts to increase or induce the activities of Phase 2 enzymes. Prochaska & Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992). In addition, these methods have been employed for isolating the compounds responsible for the inducer activities in plants and for evaluating the anticarcinogenic activities of these compounds and their synthetic analogs. Zhang *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2399-2403 (1992) and Posner *et al.*, *J. Med. Chem.* 17: 170-176 (1994).

Although inducer activity has been found in many different families of edible plants, the amounts are highly variable, depending on family, genus, species, variety, or cultivar of the plant selection and on growth and harvesting conditions. Thus, there is a need in the art to identify particular edible plants and methods of growing and preparing them that yield high levels of Phase 2 enzyme-inducer activity for chemoprotection. There is also a need for methods of growing and preparing edible plants that produce a known spectrum of specific inducers of Phase 2 enzyme activity in order to increase the efficiency with which specific carcinogens, or classes of carcinogens, are targeted for inactivation. In addition, there is a need for methods of plant breeding and selection to increase the level of Phase 2 inducer activity and to manipulate the spectrum of inducers produced in particular cultivars.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide food products and food additives that are rich in cancer chemoprotective compounds.

Another object of the present invention is to provide food products which contain substantial quantities of Phase 2 enzyme-inducers and are essentially free of Phase 1 enzyme-inducers.

It is a further object of the present invention to provide food products which contain substantial quantities of Phase 2 enzyme-inducing potential and non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

These objects, and others, are achieved by providing cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage. The cruciferous sprouts include *Brassica oleracea* varieties *acephala, alboglabra, botrytis, costata, gemmifera, gongylodes, italica, medullosa, palmifolia, ramosa, sabauda, sabellica*, and *selensia*.

Another embodiment of the present invention provides cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage, wherein the sprouts are substantially free of Phase 1 enzyme-inducing potential.

Yet another embodiment of the present invention provides a non-toxic solvent extract of cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage. The non-toxic solvent extract can be a water extract. In addition, the water extract can comprise a cruciferous vegetable, such as a cruciferous vegetable of the genus *Raphanus*, comprising an active myrosinase enzyme.

Another embodiment of the present invention provides a food product comprising cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage; extracts of the sprouts or cruciferous seeds; or any combination of the sprouts or extracts.

A further embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of a food product comprising cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage.

Another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce said sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates. The cruciferous sprouts include *Brassica oleracea* varieties *acephala, alboglabra, botrytis, costata, gemmifera, gongylodes, italica, medullosa, palmifolia, ramosa, sabauda, sabellica*, and *selensia*.

A further embodiment of the present invention provides a food product comprising sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at

least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days from growth of seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates; extracts of the sprouts or cruciferous seeds; or any combination of the sprouts or extracts.

Yet another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates and are substantially free of Phase 1 enzyme-inducing potential.

Another embodiment of the present invention provides a non-toxic solvent extract of cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates. The non-toxic solvent extract can be a water extract. In addition, the water extract can comprise a cruciferous vegetable, such as a cruciferous vegetable of the genus *Raphanus*, comprising an active myrosinase enzyme.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when

measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of a food product comprising sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

A further embodiment of the present invention provides a method of preparing a food product rich in glucosinolates, comprising germinating cruciferous seeds, with the exception of cabbage, cress, mustard and radish seeds, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts. The cruciferous sprouts include *Brassica oleracea* varieties *acephala, alboglabra, botrytis, costata, gemmifera, gongylodes, italica, medullosa, palmifolia, ramosa, sabauda, sabellica*, and *selensia* and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Yet another embodiment of the present invention provides a food product rich in glucosinolates made by germinating cruciferous seeds, with the exception of cabbage, cress, mustard and radish seeds, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts.

Yet another embodiment of the present invention provides a method of preparing a food product comprising

extracting glucosinolates and isothiocyanates from cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage, with a non-toxic solvent and recovering the extracted glucosinolates and isothiocyanates. Myrosinase enzyme, or a vegetable, such as *Raphanus* species, containing the enzyme is mixed with the cruciferous sprouts, the extract, or both the sprouts and the extract.

An embodiment of the present invention provides a method of preparing a food product rich in glucosinolates, comprising germinating cruciferous seeds having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and which contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts. The seeds may be *Brassica oleracea*, including the varieties *acephala, alboglabra, botrytis, costata, gemnifera, gongylodes, italica, medullosa, palmifolia, ramosa, sabauda, sabellica*, and *selensia*.

Yet another embodiment of the present invention provides a food product rich in glucosinolates made by germinating cruciferous seeds having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and which contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and either harvesting sprouts at the 2-leaf stage to form a food product comprising a plurality of sprouts. The nutritional product contains non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

A further embodiment of the present invention provides a method of preparing a food product comprising extracting glucosinolates and isothiocyanates with a solvent from cruciferous seeds, sprouts, plants or plant parts, wherein seeds that produce the sprouts, plants or plant parts producing sprouts having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth and wherein the seeds, sprouts, plants or plant parts have non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and recovering the extracted glucosinolates and isothiocyanates. The non-toxic extraction solvent can be water. Myrosinase enzyme, or a vegetable, such as *Raphanus* species, containing the enzyme is mixed with the cruciferous sprouts, seeds, plants, plant parts or extract, or any combination thereof.

A further embodiment of the present invention provides a method of reducing the level of carcinogens in mammals, comprising administering cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts.

Yet another embodiment of the present invention provides a method of reducing the level of carcinogens in mammals, comprising administering cruciferous sprouts having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Another embodiment of the present invention provides a method of preparing a food product by introducing cruciferous seeds, having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when

measured after 3 days of growth from seeds that produce the sprouts and non-toxic levels of indole glucosinolates and goitrogenic hydroxybutenyl glucosinolates, into an edible ingredient.

A further embodiment of the present invention provides a method of extracting glucosinolates and isothiocyanates from plant tissue which comprises homogenizing the plant tissue in an excess of a mixture of dimethyl sulfoxide, acetonitrile, and dimethylformamide (DMF/ACN/DMSO) at a temperature that prevents myrosinase activity.

Another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the ratio of monofunctional to bifunctional inducers is at least 20 to 1.

Another object of the present invention is to provide a food product supplemented with a purified or partially purified glucosinolate.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the total inducing potential of organic solvent extracts of broccoli and daikon cultivars as a function of age.

Figure 2 shows the high resolution NMR spectra of isolated glucosinolates obtained from hot aqueous extracts of 3-day old Saga broccoli sprouts.

DETAILED DESCRIPTION

I. Definitions

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

A **bifunctional inducer** is a molecule which increases activities of both Phase 1 enzymes such as cytochromes P-450 and Phase 2 enzymes and requires the participation of Aryl hydrocarbon (*Ah*) receptor and its cognate Xenobiotic Response Element (*XRE*). Examples include flat planar aromatics such as polycyclic hydrocarbons, azo dyes or 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD).

A **chemoprotector** or **chemoprotectant** is a synthetic or naturally occurring chemical agent that reduces susceptibility in a mammal to the toxic and neoplastic effects of carcinogens.

A **food product** is any ingestible preparation containing the sprouts of the instant invention, or extracts or preparations made from these sprouts, which are capable of delivering Phase 2 inducers to the mammal ingesting the food product. The food product can be freshly prepared such as salads, drinks or sandwiches containing sprouts of the instant invention. Alternatively, the food product containing sprouts of the instant invention can be dried, cooked, boiled, lyophilized or baked. Breads, teas, soups, cereals, pills and tablets, are among the vast number of different food products contemplated.

Inducer activity or **Phase 2 enzyme-inducing activity** is a measure of the ability of a compound(s) to induce

Phase 2 enzyme activity. In the present invention, inducer activity is measured by means of the murine hepatoma cell bioassay of QR activity *in vitro*. Inducer activity is defined herein as QR inducing activity in Hepa 1c1c7 cells (murine hepatoma cells) incubated with extracts of sprouts, seeds or other plant parts untreated with myrosinase. Inducer activity is measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates. Typically 10,000 Hepa 1c1c7 cells are introduced into each well. Hepatoma cells are grown for 24 hours and a plant extract containing microgram quantities of fresh plant tissue is serially diluted across the microtiter plates into fresh culture medium containing 0.15 ml αMEM culture medium amended with 10% Fetal Calf Serum (FCS) and streptomycin and penicillin. The cells are further incubated for 48 hours. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) is measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and related to its protein concentration. Quantitative information on specific activity of QR is obtained by computer analysis of the absorbances. One unit of inducer activity is the amount that when added to a single microtiter well doubles the QR activity. (*See* Prochaska and Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992)).

Inducer potential or **Phase 2 enzyme-inducing** potential is a measure of the combined amounts of inducer activity in plant tissue provided by isothiocyanates, plus glucosinolates that can be converted by myrosinase to isothiocyanates. Glucosinolates are not themselves inducers of mammalian Phase 2 enzymes, whereas isothiocyanates are inducers. Inducer potential therefore is defined herein as QR activity in murine 1c1c7 hepatoma cells incubated with myrosinase-treated extracts of the sprouts, seeds or other plant parts. In the present invention therefore inducer potential is measured by means of the murine hepatoma cell bioassay of

QR activity *in vitro* as described above. Inducer potential is measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates. Typically, 10,000 Hepa 1c1c7 cells are introduced into each well. Hepatoma cells are grown for 24 hours and a plant extract containing microgram quantities of fresh plant tissue is serially diluted across the microtiter plates into fresh culture medium containing 0.15 ml αMEM culture medium amended with 10% Fetal Calf Serum (FCS) and streptomycin and penicillin. Myrosinase (6 units/ml plant extract) is added to the plant extract. Myrosinase is purified by modification of the technique of Palmieri *et al.*, *Anal. Biochem.* 35: 320-324 (1982) from 7 day old Daikon sprouts grown on agar support containing no added nutrients. Following 234-fold purification, the myrosinase had a specific activity of 64 units/mg protein [unit = amount of enzyme required to hydrolyze 1 μmol sinigrin/min]. Plant extract is diluted 200-fold into the initial wells of the microtiter plate followed by 7 serial dilutions. The cells are further incubated for 48 hours. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) is measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and related to its protein concentration. Quantitative information on specific activity of QR is obtained by computer analysis of absorbances. One unit of inducer potential is the amount that when added to a single microtiter well doubles the QR activity. (*See* Prochaska and Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992)).

A **monofunctional inducer** increases the activity of Phase 2 enzymes selectively without significantly altering Phase 1 enzyme activities. Monofunctional inducers do not depend on a functional *Ah* receptor but enhance transcription of Phase 2 enzymes by means of an Antioxidant Responsive Element (ARE).

A cruciferous **sprout** is a plant or seedling that is at an early stage of development following seed germination. Cruciferous seeds are placed in an environment in which they germinate and grow. The cruciferous sprouts of the instant invention are harvested following seed germination through and including the 2-leaf stage. The cruciferous sprouts of instant invention have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential at 3-days following incubation under conditions in which cruciferous seeds germinate and grow.

II. Description

A major mechanism of protection provided by fruits and vegetables in reducing the cancer incidence in humans depends on minor chemical components which, when delivered to mammalian cells, elevate levels of Phase 2 enzymes that detoxify carcinogens. It has now been discovered that the anticarcinogenic activity of certain edible plants can be increased. Plants such as *Brassica oleracea* variety *italica* (broccoli) are normally not harvested until they form heads. By growing these plants only to the seedling or sprout stage, that is between the onset of germination and the 2-leaf stage, the levels of inducers of enzymes that detoxify carcinogens and protect against cancer can be increased at least five-fold over those found in commercial stage vegetables of the same cultivars. Often increases of between 10 and 1000-fold have been observed.

Harvesting plants at an early seedling or sprout stage, or otherwise arresting their growth, leads to the greatest inducer potential and yields a food product of a type to which consumers are already accustomed. The Phase 2 enzyme-inducing potential of such sprouts may be as much as several hundred times higher than that observed in adult, market stage vegetables obtained from the same seeds. Thus it is possible that humans can consume the same quantities of inducer potential by

eating relatively small quantities of sprouts, rather than large quantities of market-stage vegetables.

It has now been found that most of the inducer potential of crucifer plants is due to their content of isothiocyanates and their biogenic precursors, glucosinolates. Glucosinolates are converted to isothiocyanates by the enzyme myrosinase which is a thioglucosidase. Normally myrosinase and glucosinolates are separated in the cell and if the cell is damaged, with loss of compartmentalization, myrosinase comes into contact with glucosinolates, which are then converted to isothiocyanates.

In order to screen large numbers of edible plants and to evaluate the effects of environmental perturbation on Phase 2 enzyme-inducer potential in those vegetables, it was necessary to improve upon the previously described techniques for homogenization and extraction of those vegetables. Techniques initially described for the extraction of Phase 2 inducers from vegetables involved homogenization of the vegetables in cold water, lyophilization, extraction of the resultant powder with acetonitrile, filtration and evaporative concentration, Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992).

Following identification of sulforaphane as the principal Phase 2 inducer from broccoli, comparative extractions were performed into hot 80% methanol, yielding similar inducer activity as the aforementioned acetonitrile extracts. When myrosinase was added to these hot methanol extracts in which glucosinolates are freely soluble, there was a dramatic enhancement of the Phase 2 inducer activity of these extracts (data summarized in Table 1). The deliberate conversion of these glucosinolates to isothiocyanates using exogenous myrosinase thus gave a better index of the inducers for Phase 2 enzymes of the vegetables tested. It was thus

clear that the majority of the potential Phase 2 inducers in crucifers was usually present in whole plants as the glucosinolate precursors of isothiocyanates.

The preponderance of glucosinolates and the rapidity with which, upon wounding of cruciferous plant tissue, glucosinolates are converted to isothiocyanates, led to the development of an improved extraction procedure. By manipulation of solvent mixtures and of the water activity of fresh vegetable/solvent homogenates, a procedure was developed that permits both glucosinolate and isothiocyanate quantification from the same, non-concentrated sample. In addition to being the rate-limiting step in an extraction protocol, evaporative concentration allows volatile inducers to escape detection. The improved procedure is both simple and efficient, requiring only that the plant sample be completely homogenized in solvent. Using this technique, the present inventors have thus been able to demonstrate dramatic increases in the recovery of inducer activity and inducer potential from cruciferous vegetables over previously described techniques.

If fresh-picked vegetables are promptly and gently harvested, directly into organic solvents comprising a mixture of DMF/ACN/DMSO and a temperature that prevents myrosinase activity, both glucosinolates and isothiocyanates are efficiently extracted into the organic solvent mixture. Preferably, the DMF, ACN and DMSO are mixed in equal volumes. However, the volumes of the three solvents in the mixture can be varied to optimize extraction of specific glucosinolates and isothiocyanates from any plant tissue. The temperature of the extraction mixture is preferably less than 0°C, and most preferably less than -50°C. The temperature of the extraction solvent must be kept above freezing. At the same time the enzyme myrosinase, which invariably accompanies these constituents in the plants and rapidly converts glucosinolates into isothiocyanates, is

inactive. Such extracts typically contain high quantities of glucosinolates and negligible quantities of isothiocyanates. The *in planta* myrosinase activity varies between different plant species.

Glucosinolates are not themselves inducers of mammalian Phase 2 enzymes, whereas isothiocyanates are monofunctional inducers in the murine hepatoma cell bioassay of QR activity. The inducer potential, as distinct from inducer activity, of plant extracts can be measured by adding purified myrosinase, obtained from the same, or other plant sources, to the assay system.

Glucosinolates are converted at least partially to isothiocyanates in humans. If, however, it is desirable to accelerate this conversion, broccoli or other vegetable sprouts, high in glucosinolates, can be mixed with myrosinase. The mixture can be in water, or some other non-toxic solvent that does not inactivate myrosinase. The myrosinase can be from a partially purified or purified preparation. Alternatively, the myrosinase can be present in plant tissue, such as a small quantity of crucifer sprouts rich in myrosinase, including *Raphanus sativus* or daikon. Such a preparation can be used to produce a "soup" for ingestion that is high in isothiocyanates and low in glucosinolates. Inducer potential can be measured using a multiwell plate screen with murine hepatoma cells for *in vitro* measurement of QR specific activity as described above.

The ratio of monofunctional to bifunctional inducer activity of plant tissue is measured by bioassaying plant extracts, as described above, not only in wild-type Hepa 1c1c7 cells, but also, in mutants designated c1 and BP^{r}c1 that have either defective *Ah* receptors or defective cytochrome P_1-450 genes, respectively. Prochaska and Talalay, *Cancer Research* 48: 4776-4782 (1988).